

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 0 407 122 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
02.10.1996 Bulletin 1996/40

(51) Int Cl.⁶: C12P 21/02, C07K 14/00,
A61K 38/00, A61K 47/48,
A61K 39/395

(21) Application number: 90307203.1

(22) Date of filing: 02.07.1990

(54) Novel modified PF4 compositions and methods of use

Modifizierte PF4-Zusammensetzung und Methoden zu deren Verwendung

Compositions de PF4 modifié et méthode d'utilisation

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(30) Priority: 06.07.1989 US 376333

(43) Date of publication of application:
09.01.1991 Bulletin 1991/02

(60) Divisional application: 95120360.3

(73) Proprietor: REPLIGEN CORPORATION
Cambridge, MA 02139 (US)

(72) Inventor: Malone, Theodore E.
Wakefield, MA 01880 (US)

(74) Representative: Perry, Robert Edward et al
GILL JENNINGS & EVERY
Broadgate House
7 Eldon Street
London EC2M 7LH (GB)

(56) References cited:
EP-A- 0 088 695 EP-A- 0 281 363
WO-A-85/04397 WO-A-86/06384

- CHEMICAL ABSTRACTS, vol. 98, no. 10, 07 March 1983, Columbus, OH, US; A. TROUET et al.: "Targeting of antitumor and antiprotozoal drugs by covalent linkage to protein carriers", p. 334, ref.no. 77997M
- CHEMICAL ABSTRACTS, vol. 112, no. 23, 04 June 1990, Columbus, OH, US; D.I. SILBERT et al.: "Glycosaminoglycans of bovine aorta endothelial cells: Identification and localization by use of a platelet factor 4-fluorescein probe", p. 300, ref.no. 213283N
- CELL., vol. 22, 1980, Cambridge, MA, US, pp. 563-570; CAWLEY & HERSCHMAN: "Epidermal growth factor-Toxin A conjugates: EGF-Ricin A is a potent toxin while EGF-Diphtheria fragment A is nontoxic"

Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description

Background of the Invention

5 Angiogenesis, the development of new capillary blood vessels, is an important process in the developing fetus and growing human. However, in healthy adults, angiogenesis occurs significantly only during wound healing and in the menstrual cycle.

10 It is now widely recognized that much of the angiogenic activity occurring in adults is pathological in nature. For example, proliferation of vascular endothelial cells and formation of new capillaries is essential for growth of solid tumors beyond a few cubic millimeters in volume (Folkman et al. [1983] Ciba Found. Symp. 100:132-149). We now understand that developing tumors secrete growth factors which stimulate neighboring endothelial cells to divide and migrate toward the tumor.

15 In addition to growth of solid tumors, other conditions involving angiogenic dysfunctions include diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation (including rheumatoid arthritis), capillary proliferation within atherosclerotic plaques, hemangiomas, and Kaposi's Sarcoma have also recently been recognized as diseases possessing characteristics of dysregulated endothelial cell division and capillary growth. These conditions along with growth of solid tumors are collectively referred to as "angiogenic diseases" (Folkman, J., and M. Klagsbrun [1987] Science 235:442-447).

20 In addition to angiogenic diseases, there are other conditions where endothelial cell proliferation is pathological or, at least, unwanted. For example, endometriosis is characterized by the abnormal proliferation and positioning of certain endothelial cells which normally line the inner wall of the uterus. Control of the angiogenic process could help to prevent or alleviate endometriosis. Also, prevention of endothelial cell growth in the uterus could be a means of birth control.

25 Endothelial cell growth is associated with wound healing. This growth is undesirable during extended surgical proceedings and where excessive scar formation may occur. Therefore, a means of controlling endothelial cell proliferation would help prevent or reduce unwanted scar formation.

30 The mechanism of angiogenesis and endothelial cell proliferation has not been completely characterized. It has been established that mast cells accumulate at a tumor site before new capillary growth occurs; however, mast cells alone cannot initiate angiogenesis. Heparin, a mast cell product, has been shown to significantly stimulate the capillary endothelial cell migration which is necessary for angiogenesis (Folkman, J. [1984] Angiogenesis: Initiation and Modulation. In Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects, G.L. Nicolson and L. Milas, eds. Raven Press, New York, pp. 201-208).

35 Several substances are known to have the capability of inhibiting endothelial cell growth in vitro. One of the most extensively studied inhibitors of endothelial cell growth is protamine, which is a protein found only in sperm. Protamine has been shown to inhibit tumor angiogenesis and subsequent tumor growth (Taylor, S. and J. Folkman [1982] Nature 297:307-312). Protamine's anti-angiogenesis activity has been attributed to its well-known capacity to bind heparin (Taylor and Folkman [1982], supra). Clinical experiments with protamine have not been pursued because of the toxicity associated with protamine injection. Protamine, which is usually isolated from salmon sperm, is known to be antigenic in humans, and anaphylactic reactions to this protein have been observed with secondary exposures.

40 At least two other compounds have been studied in regard to their heparin-binding activity: platelet factor 4 (PF4) and major basic protein. Major basic protein has demonstrated heparin-binding activity but is of little practical utility because of its high toxicity.

45 Platelet factor 4 is a well-known protein which has been completely sequenced (Deuel, T.F., P.S. Keim, M. Farmer, and R.L. Henrikson [1977] Proc. Natl. Acad. Sci. USA 74(6):2256-2258). It is a 70-residue secreted platelet protein with a molecular weight of approximately 7.8 Kd. Although there is evidence of heparin binding activity and some indications of anti-angiogenesis activity (Folkman [1984], supra), PF4 has never been shown to have clinical utility.

50 A compound which has been described as "oncostatin A," and which appears to be the same, or similar to, native PF4, has been implicated as effecting the growth of tumors (U.S. Patent Nos. 4,645,828 and 4,737,580; both issued to Twardzik et al.). However, the effects reported in these patents pertain to slowly growing human cancer cells in immunodeficient mice. The results of these experiments cannot be reliably extrapolated to predict the effect of rapidly growing tumors which are native to the host animal. Furthermore, the experiments reported in these patents in no way predict or disclose any angiostatic properties.

55 Various peptides from PF4 have been purified and their properties studied. None has been shown to have any role in the inhibition of angiogenesis. It is known that the C-13 peptide of PF4 is chemotactic for neutrophils and monocytes (Deuel, T.F., R.M. Senior, D. Chang, G.L. Griffin, R.L. Henrikson, and E.T. Kaiser [1981] Proc. Natl. Acad. Sci. USA 78:4585-4587; Osterman, D.G., G.L. Griffin, R.M. Senior, E.T. Kaiser, and T.H. Deuel [1982] Biochem. and Biophys. Res. Comm. 107(1):130-135). It is significant to note that the infiltration of monocytes would be expected to stimulate the proliferation and migration of local endothelial cells, by the secretion of angiogenic factors. Thus, peptides

of PF4 could be expected to stimulate, rather than inhibit, angiogenesis.

There is a significant and very long-standing need to locate an effective and non-toxic inhibitor of angiogenesis and endothelial cell proliferation. Angiogenesis plays a major role in the initiation and progression of widespread catastrophic illnesses, including cancer. An effective, non-toxic agent which can be administered locally and/or systemically to treat these illnesses would be highly advantageous and has long eluded identification.

EP-A-0281363 discloses conjugates of PF4 with an immunogenic potentiator, e.g. an antigen or particle, for the production of monoclonal antibodies or polyclonal sera. It also discloses covalently coupling a cytotoxic agent such as a toxin A-chain fragment or a targeting molecule such as a hormone or antibody to the leader sequence.

Summary of the Invention

According to the present invention, a polypeptide conjugate comprises platelet factor 4 (PF4), or an angiogenesis-inhibiting fragment or mutant thereof, is conjugated to a second molecule selected from fluorophores, chelators, carrier molecules, antibodies, toxins and cell receptor molecules or complementary ligands thereof, and is used for the manufacture of a medicament for use in inhibiting angiogenesis or endothelial cellular proliferation.

The conjugates may be obtained by chemical modification of PF4 or recombinant PF4 (rPF4). For example, PF4 can be modified through its free amino groups with fluorescein-isothiocyanate and retain the capability of inhibiting angiogenic activity and endothelial cell proliferation. Similar modifications can be made with PF4 analogues, mutants or fragments.

A further aspect of the present invention is the targeting of the biological activity of PF4 to specific locations where that activity is needed. This can be done by conjugating PF4 (or an appropriate fragment, analogue or mutant) to a monoclonal or polyclonal antibody, carrier protein, cell receptor molecule, or other binding protein sequence. In addition to treating angiogenic disorders and inhibiting endothelial cell proliferation, modified PF4 can also be used to target toxins to specific cell populations. Various other modifications of PF4 and related compounds are described here. These modifications can be made in order to enhance biological activity of otherwise increase the utility of the PF4 compound.

Brief Description of the Drawings

Figure 1 shows the inhibition of angiogenesis resulting from treatment with rPF4 and various related peptides.

Figure 2 compares the amino acid sequence of rPF4 with rPF4-241.

Figure 3 depicts the α -helical configurations of rPF4 and rPF4-241.

Figure 4 compares the inhibition of angiogenesis resulting from treatment with rPF4 and rPF4-241.

Figure 5 shows inhibition of human endothelial cell proliferation by rPF4 and rPF4-241.

Figure 6 compares the inhibition of human umbilical vein endothelial cell proliferation resulting from treatment with rPF4 or rPF4-241.

Figure 7 shows the ability of rPF4 to inhibit tumor growth.

Figure 8 depicts the possible chemical structure of the C-terminal end of FrPF4.

Figure 9 shows inhibition of human endothelial cell proliferation by FrPF4.

Figure 10 shows inhibition of human endothelial cell proliferation by FrPF4-241.

Figure 11 shows inhibition of tumor growth by FrPF4.

Detailed Description of the Invention

The subject invention concerns the discovery that PF4, rPF4, and fragments and analogs of these compounds can be chemically modified to create new compounds with highly desirable characteristics. For example, chemical modification of rPF4 and its fragments has resulted in the identification of compounds which show surprising ability to inhibit angiogenic activity as well as the capability to inhibit endothelial cell proliferation. One specific chemical modification which resulted in altered biological properties involved modification of the free amino groups of rPF4 with fluorescein-isothiocyanate (FITC). The resulting adduct, FrPF4, lacks heparin binding activity because of modification of lysine residues within the heparin binding domain but, surprisingly, retains the ability to inhibit angiogenesis as well as suppress HUVEC proliferation in vitro.

Angiostatic activity is also found in PF4 fragments and mutants which have been modified with the bulky and hydrophobic fluorescein moiety. In addition to their biological activity, the FITC-labeled PF4 sequences are useful for visual detection of PF4 molecules. Furthermore, the ability to modify PF4 and its fragments with large moieties without loss of the relevant biological activity provides a basis for conjugating PF4, its fragments, mutants, or derivatives with toxins, monoclonal antibodies, polyclonal antibodies, fluorophores, cell receptor molecules, non-proteinaceous biological effector molecules, chelators, carrier proteins, and other large entities.

One of the uses of the compounds described here is in the treatment of angiogenic diseases. As used in this application, the term "angiogenic disease" refers to growth of solid tumors, and other conditions involving angiogenic dysfunctions including diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation (including rheumatoid arthritis), capillary proliferation within atherosclerotic plaques, hemangiomas, and Kaposi's Sarcoma. The subject invention also concerns the use of rPF4 and PF4 fragments, analogs, and mutants for treatment of diseases of dysregulated endothelial cell proliferation.

As used in this application, the term "analog" refers to compounds which are substantially the same as another compound but which may have been modified by, for example, adding additional amino acids or side groups. "Mutants" as referred to in this application refers to amino acid sequences which are substantially the same as another sequence but which have different amino acids at certain locations in the amino acid sequence. "Fragments" refer to portions of a longer amino acid sequence.

The subject invention embraces the specific amino acid sequences and other compositions which are specifically exemplified. The subject invention further embraces analogs and mutants of these sequences, as well as fragments of the sequences, and analogs and mutants of the fragments. These analogs, mutants, and fragments are embraced within the subject invention so long as the analog, fragment, or mutant retains substantially the same relevant biological activity as the originally exemplified compound. For example, it is well within the skill of a person trained in this art to make conservative amino acid substitutions. These substitutions are discussed in more detail below. To the extent that these substitutions do not substantially alter the relevant biological activity, then the resulting compounds fall within the scope of the subject invention. The term "relevant biological activity" refers to the activity of interest for a particular application of a compound. For example, several uses of PF4 are discussed below. These uses include inhibition of angiogenesis and endothelial cell proliferation. When PF4 is being used in these ways then "analogs" would refer to compounds where PF4 has been modified (by a conservative amino acid substitution, for example) without substantially altering the compound's ability to inhibit angiogenesis or endothelial cell proliferation. Conservative amino acid substitutions are only one example of the type of modifications which are within the scope of the subject matter of this invention.

The subject invention rises from the unexpected discovery that chemically modified rPF4 inhibits *in vivo* capillary formation and embryonic neovascularization. It has been determined that full length recombinant PF4 inhibits growth factor-dependent human endothelial cell proliferation *in vitro*. Significantly, it has also been determined that the angiogenesis-inhibiting activity of PF4 is retained by synthetic peptides corresponding to sequences of PF4 as small as 13 amino acids in length. In particular, a synthetic peptide of 13 amino acids corresponding to the carboxy terminus of PF4 (C-13) has displayed potent angiostatic activity.

The activity of the C-13 peptide is especially surprising in light of its inability to affect the anticoagulant activity of heparin. The use of the C-13 peptide offers several advantages over whole rPF4 such as reduced dosage (weight basis), reduced likelihood of antigenicity, and greater likelihood of effectiveness in novel dosage forms.

The C-13 peptide of PF4 also retains the ability to prevent Con-A induced immunosuppression in mice, an activity which is unaffected by heparin and probably independent of the ability of the peptide to inhibit angiogenesis.

It is well understood that angiogenesis is required for solid tumors to grow beyond a few cubic millimeters. Thus for the treatment of solid tumors, use of rPF4, or modifications thereof, to cause tumor rejection by inhibiting angiogenesis presents a novel and highly advantageous means of therapy. The fact that the C-13 peptide inhibits angiogenesis without affecting the anticoagulant activity of heparin demonstrates that this small peptide would also have the benefit of not interfering with concurrent anticoagulant therapy. Additionally, small peptides are generally less antigenic than larger proteins, and, thus, the PF4 fragments can be used advantageously for oral and transdermal administration. These types of delivery are particularly useful in the treatment of gastrointestinal capillary proliferation (e.g., Kaposi's Sarcoma) and skin lesions, respectively. Intralesional, as well as systemic, administration of PF4 fragments are also appropriate for treatment of these conditions. Topical or aerosol administration of PF4 fragments is appropriate for skin or pulmonary lesions, respectively (e.g., Kaposi's sarcoma and lung cancer).

An analog of PF4 which exhibits enhanced ability to inhibit angiogenesis has been synthesized. This analog, known as rPF4-241, was created by cassette mutagenesis of a synthetic PF4 gene whereby four lysine residues of the carboxy terminus of PF4 were converted to two Gln-Glu couplets in order to eliminate heparin binding activity while retaining the α -helical secondary structure. If rPF4-241 (or FrPF4-241) is administered intralesionally, it can be applied such that the dosage is between about 1 μ g/lesion and about 4 mg/lesion. For systemic administration, the dosage of rPF4-241 (or FrPF4-241) can be between 0.5 mg/kg of body weight and about 100 mg/kg of body weight. Similar and higher dosages can be used for the administration of native sequence rPF4 (or FrPF4) as well as peptide fragments. For example, dosages of rPF4 (or FrPF4) and fragments thereof may be twice that of rPF4-241 (or FrPF4-241) or higher.

The compounds of the subject invention can be combined with a suitable pharmaceutical carrier. For example, FrPF4 or FrPF4-241 can be formulated in physiologically acceptable carriers, such as phosphate buffered saline, distilled water, excipients, or the like, or may be administered neat.

Materials and Methods

Chicken Chorioallantoic Membrane (CAM) Assay. Fertile eggs were incubated in a stationary position for 3 days at 37°C and 70-80% relative humidity. During this time, the embryo rose to the upper surface of the egg contents. At the beginning of the 4th day, the eggs were cracked without inversion and carefully deposited into sterile plastic petri dishes such that the embryo remained on the upper surface. The shell-free eggs were incubated for an additional 72 hours at 37°C, under an atmosphere containing 2.5-3.5% CO₂ after which the growing embryos developed a recognizable CAM. Discs, made by mixing test samples with 1% (w/v) methylcellulose were dried and placed on the CAM between major veins and approximately 0.5 cm from the embryo. Following another 48 hour incubation at 37°C (2.5-3.5% CO₂), the samples were scored for their ability to inhibit angiogenesis. Inhibition appears as an avascular zone surrounding the implant and can often include elbows formed by veins avoiding the disc and a reduced number of capillaries in the region of the implant.

Endothelial Cell Proliferation Assay. Human umbilical vein endothelial cells were cultured in Medium 199 (Gibco) containing 10% (v/v) fetal bovine serum (FBS), 150 mcg/ml endothelial cell growth supplement (ECGS) and 5 units/ml heparin at 37°C and 4-5% CO₂. Every 3-4 days, the cultures were harvested by trypsin treatment, diluted, replated, and grown to confluence. Prior to the start of an experiment, the cells were centrifuged and resuspended in heparin-free media and incubated with the test substance for 3 days under standard culture conditions. At the end of the incubation period, the cells were harvested and counted. Statistical significance between means was determined by a standard Student t-test for unpaired data.

rPF4 Production. Recombinant PF4 was produced in *E. coli* as an N-terminal fusion protein containing a methionine immediately preceding the PF4 sequence. The insoluble fusion protein was cleaved with cyanogen bromide treatment and purified by heparin agarose affinity chromatography. The isolated protein was buffer exchanged into 20 mM sodium acetate, pH 4.0, and either frozen or lyophilized for storage.

Production of Peptides. Peptides were prepared by standard solid phase synthesis procedures, cleaved from the solid support and deblocked, and purified by reverse phase HPLC.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1

Chicken eggs, prepared as described above, were treated with discs containing several concentrations of recombinant PF4 or peptides derived from the sequence of PF4. rPF4 and C-terminal peptides as small as 13 amino acids inhibited angiogenesis on the CAM (Figure 1). In each case, the inhibition was dose-dependent and the response approximately equivalent (molar basis) for the inhibitors containing the C-terminal region of PF4. An N-terminal peptide of PF4 (N-29) did not inhibit angiogenesis even at the highest concentration tested, suggesting that all of the anti-angiogenic activity of PF4 is probably associated with the C-terminal portion of the molecule. Since the C-terminus of PF4 is rich in lysine, polylysine was tested in this assay system and found not to cause inhibition at 6.5 nmol dosages.

Example 2

The lysine rich region of PF4 (residues 61-66) is also the domain associated with the binding of heparin by PF4. Heparin is known to play a role in modulating angiogenesis, which can also be affected by protamine, another well characterized heparin-binding protein. To assess the ability of PF4-based synthetic peptides to bind heparin, we assayed the activity of coagulation-cascade enzymes which are inhibited by heparin. Protamine and platelet factor 4 are able to prevent the heparin inhibition of thrombin and Factor Xa at approximately equimolar concentrations. The 41 amino acid C-terminal peptide of PF4 (C-41) prevented heparin inhibition less effectively, but the C-13 peptide was unable to prevent the inhibition of thrombin even at concentrations ten times that of an effective level of rPF4. This unexpected finding suggests that the C-13 peptide inhibits angiogenesis by some method other than heparin binding.

Example 3

Many angiostatic agents act by direct inhibition of endothelial cell proliferation. Endothelial cell division and growth is tightly controlled and strictly dependent on the presence of growth factors. We evaluated the ability of rPF4 and related peptides to inhibit growth factor-stimulated human endothelial cell proliferation *in vitro*. rPF4 significantly inhibited endothelial cell growth in a dose-dependent fashion at a concentration as low as 10 µg/ml. Inhibition was complete at 25 µg/ml in the heparin-deficient medium employed here.

Example 4

To assess the importance of the heparin binding activity of PF4 in the inhibition of endothelial cell proliferation, cells were incubated in media containing or lacking 5 units/ml heparin. The presence of heparin stimulated proliferation of these cells during the three day incubation of this experiment. rPF4 significantly inhibited both control (100%) and heparin stimulated (45%) endothelial cell growth (Table I).

Table 1.

Attenuation of rPF4 inhibition of endothelial cell growth by heparin.			
Addition	rPF4		%
	--	50mcg/ml	Inhibition ^a
--	14.4 ± 2.5	^b 6.0 ± 0.6	~100
5 u/ml heparin	18.9 ± 1.2	^b 14.0 ± 0.4	45

^aBased on seeding of 8×10^4 cells/well

^bSignificantly different from appropriate control ($p < 0.005$)

Example 5 - Construction of rPF4-241

Cassette mutagenesis of a synthetic PF4 gene was used to convert the four lysine residues at the carboxy terminus of PF4 to two Gln-Glu couplets (see Figure 2). This construction apparently retains the α -helical secondary structure (Figure 3) for this region of the molecule with the concurrent loss of heparin binding activity.

The gene for rPF4-241 was expressed as a fusion protein in *E. coli* with the same N-terminal amino acid sequences as with the parent rPF4 molecule. The protein was cleaved from the *E. coli* fusion peptide by CNBr and formic acid and purified to near homogeneity by DEAE-sepharose chromatography. The protein was reactive with polyclonal antibodies to PF4 and was determined to possess the appropriate modifications by amino acid analysis. Significantly, the purified mutant protein lacked heparin-binding activity in the Factor Xa inhibition assay.

The substitutions described here can be made with the peptide fragments as well as with the full length PF4 molecule. For example, C-13-241 has the following sequence:

Pro-Leu-Tyr-Gln-Glu-Ile-Ile-Gln-Glu-Leu-Leu-Glu-Ser

Example 6 - Inhibition of angiogenesis by rPF4-241

Purified rPF4-241 was dried in methylcellulose discs and tested for its ability to inhibit capillary growth in the chicken chorioallantoic membrane (CAM) assay. Even at the lowest concentrations tested (1.25 nmol/disc) rPF4-241 extensively inhibited angiogenesis in the CAM system (Figure 4). This inhibition was even more effective than that caused by equal concentrations of native rPF4 as suggested by larger avascular zones on the membrane. The inhibitory effect of rPF4-241 was not reversed by heparin.

Example 7 - Inhibition of human endothelial cell proliferation by rPF4-241

At concentrations where native rPF4 completely inhibits endothelial cell proliferation, mutant rPF4-241 was at least as effective in inhibiting cell growth (Figure 5). Further tests suggest that rPF4-241 was inhibitory at concentrations as low as 0.5 mcg/mL, a level at which native rPF4 has little or no effect.

In a test of inhibition of human umbilical vein endothelial cell proliferation by native rPF4 and mutant rPF4-241, the rPF4-241 was shown to be much more effective than the native rPF4 at inhibiting the proliferation of these cells. The results of this test are shown in Figure 6.

These results are remarkable in that previous theories of PF4 inhibition of angiogenesis assumed that the PF4 effects were due to heparin binding. We have designed a protein, retaining most of the structural features of native PF4 but lacking detectable heparin binding activity, which is apparently more active than native PF4 in inhibiting angiogenesis *in vivo* and endothelial cell proliferation *in vitro*. Additionally, the mutant we have designed would not be expected to interfere with heparin anticoagulant therapy.

Example 8 - Inhibition of *In Vivo* Tumor Growth

Normal C57BL/6J female mice (6-8 weeks old) were inoculated subcutaneously with 5×10^5 log phase cells of a B16-F10 melanoma tumor line. This protocol led to progressive tumor growth resulting in large (300 mm³) necrotic tumors after approximately 10 days, followed by death of untreated animals usually within three weeks of tumor inoculation.

In an experiment to test the efficacy of rPF4 in preventing *in vivo* tumor growth and angiogenesis, tumor bearing animals were divided into two groups. One group was injected with 50 µg rPF4 (native sequence) in 100 µl of 50 mM sodium phosphate, pH 6.5, 50 mM sodium chloride directly into the nascent tumor, daily, beginning one day after tumor inoculation. A control group was treated identically with carrier buffer lacking rPF4. Tumor volume was measured at regular intervals with digital calipers by laboratory personnel uninformed of the specific treatment received by each subject animal.

Within seven days of tumor inoculation, control animals possessed obvious three dimensional tumors, while rPF4-treated animals were essentially tumor-free (Figure 7). Continued treatment with rPF4 completely suppressed tumor growth under these conditions where control animal tumors became necrotic and large as seen previously with untreated mice. The same effect was observed when rPF4-241 was used as the inhibitory agent.

This finding supports the proposition that rPF4, as an inhibitor of angiogenesis, will possess clinical usefulness in the management of malignant melanoma and other cancers. Progressive growth of tumors requires new blood vessel formation which, if inhibited, may not only restrict tumor growth, but stimulate regression of existing vessels, as well as enhance other responses to malignant invasion.

The finding that rPF4 inhibition of *in vivo* tumor growth was apparent within three days of the initial inoculation (of rPF4) indicates that rPF4 acts to modulate tumor growth by local mechanisms rather than by immunomodulation which would require a longer time course. Additionally, rPF4 did not directly inhibit tumor cell growth *in vitro*. It appears, therefore, that rPF4 was modulating the host's angiogenic response to the growing tumor.

It has been shown that proteins of identified structure and function may be constructed by changing the amino acid sequence if such changes do not alter the protein secondary structure (Kaiser, E.T. and F.J. Kezdy [1984] Science 223:249-255). Thus, the subject invention includes mutants of the amino acid sequences depicted herein which do not alter the protein secondary structure, or if the structure is altered, the biological activity is retained. In particular it should be understood that conservative substitutions of amino acids may be made. For example, amino acids may be placed in the following classes: basic, hydrophobic, acidic, polar, and amide. Substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. Table 2 provides a listing of examples of amino acids belonging to each class.

Table 2.

Class of Amino Acid	Example of Amino Acids
Basic	Lys, Arg, His
Hydrophobic	Leu, Ile, Val, Phe, Trp
Acidic	Glu, Asp
Polar	Ser, Thr
Amide	Gln, Asn

In some instances, non-conservative substitutions can also be made. For example, lysine may be substituted for with any of the following amino acids: Glu, Gln, Asp, Asn, Met, Ala, Leu, and Ile. The critical factor is that these substitutions must not significantly detract from the biological activity of the rPF4 or the rPF4 fragment.

Example 9 - Modification of PF4 and rPF4-241 with Fluorescein-Isothiocyanate (FITC)

Purified rPF4 or rPF4-241 (5 mg in 50 mM Na₂CO₃, pH 9.3, 25 mM NaCl) was treated with 5 mg of fluorescein isothiocyanate in a volume of 5 ml to modify the free amino groups. After incubation for 3 hours at room temperature in the dark, the labeled protein (FrPF4 or FrPF4-241) was separated from unbound FITC by gel filtration and dialyzed into 50 mM acetic acid. A possible structure of the C-terminus of FrPF4 is shown in Figure 8.

Example 10 - Inhibition of Angiogenesis by Fluorescein-Isothiocyanate-Conjugated rPF4

FrPF4 was tested for activity in the CAM assay as described above. Although FrPF4 lacked heparin binding activity,

it retained full activity as an inhibitor of angiogenesis on the CAM. The results of these assays are shown in Table 3.

Table 3.

Activity of FrPF4 in the CAM assay.		
Amount per disc (μg)	Inhibition (%)	
	rPF4	FrPF4
0	0	0
10	22	17
25	33	33

Example 11 - Inhibition of Endothelial Cell Proliferation by FrPF4 and FrPF4-241

FrPF4 and FrPF4-241 were tested separately to determine their ability to inhibit endothelial cell proliferation. HUVE cells were tested for their sensitivity to FrPF4 as described above except that [³H]-thymidine was added to the cultures 24 hours after the addition of FrPF4. The cultures were then incubated an additional 6 hours. Cells were harvested, washed, and radioactive thymidine incorporation into DNA was measured.

As shown in Figure 9, FITC-conjugated rPF4 was very effective, even at low dosages, in inhibiting DNA synthesis in human umbilical vein endothelial cells and therefore inhibiting cell proliferation. Similar results were obtained using FrPF4-241. In this case, the inhibition of HUVE cell proliferation with increasing concentrations of rPF4-241 was tested using the Endothelial Cell Proliferation Assay as described above. The results of experiments using FrPF4-241 are shown in Figure 10.

Example 12 - Inhibition of *In vivo* Tumor Growth by FITC-rPF4

B-16 Melanoma tumors were grown in C57BL6/J mice as described previously. Treatment was begun 24 hours following implantation of tumor cells (Day 1) and consisted of 25 μg/day of FrPF4 in 100 μl of sodium acetate buffer, pH 4.0. Control mice were injected with 25 μg/day of FITC labeled cytochrome-C in the same buffer. A statistically significant suppression of tumor growth by FrPF4 was observed by Day 11 (Figure 11).

Example 13 - Delivery of PF4 Activity to Specific Sites

For treatment of certain conditions, it is sometimes advantageous to direct biological activity to a specific location. For example, in order to inhibit solid tumor growth, it may be desirable to send PF4, or an analog with angiostatic properties, directly to the tumor site. This can be accomplished by coupling the PF4 (or analog) to an appropriate antibody, preferably a monoclonal antibody. The monoclonal antibody, which can be produced using techniques that are well-known in the art, will selectively seek out the target site. As the antibody moves to the desired location, it brings with it the PF4. Thus, the PF4 activity can be concentrated at a specific location.

General means of conjugating antibodies to polypeptides such as PF4 are well known to those skilled in the art and are discussed, for example, in U.S. Patent Nos. 4,671,958 (Rodwell et al.) and 4,792,447 (Uhr et al.). The PF4 may also be targeted to specific locations via analogous conjugation with binding proteins (e.g., thrombospondin or fibroblast growth factor), cell receptor molecules (e.g., CD4, lymphocyte function associated antigen-1 [LFA-1], and von Willebrand Factor [vWF]) or the complementary ligands, and non-proteinaceous biological effector molecules (e.g., ICAM-1, tumor-associated-antigens, and prostaglandins).

For example, the monoclonal antibody, or other moiety, can be associated with PF4 at one or both pairs of lysine residues located near the carboxy terminus of PF4. By associating the monoclonal antibodies at these residues, the angiostatic activity is retained while heparin binding is eliminated. Also, other amino acid residues may be substituted for the lysine residues before conjugation with appropriate moieties at these and other positions. Therefore, the compounds described here can be represented as follows:



where:

- (a) **A** represents all or part of the polypeptide sequence consisting of residues 1 through 57 of PF4. **A** may or may not be present on said hybrid polypeptide;
- (b) **B**, **C**, **D**, and **E** can be any amino acid; and
- (c) **F**, **G**, **H**, and **I** are selected from the group consisting of monoclonal antibodies, polyclonal antibodies, fluorescein-isothiocyanate, fluorophores, toxins, cell receptor molecules, non-proteinaceous biological effector molecules, and chelators; at least one of the moieties designated **F**, **G**, **H**, and **I** must be present on said hybrid polypeptide.

In the above representation of the compounds described here, the vertical lines represent chemical bonding interactions as do the spaces between the amino acids on the horizontal line. The existence of specifically illustrated moieties associated at **B**, **C**, **D**, and **E** does not exclude the possibility of conjugation occurring at other residues.

Example 14 - Conjugation of Carrier Proteins to PF4

It may be desirable to increase the circulating half-life of PF4 to improve its effectiveness as a systemically active angiostatic complex for tumor and angiogenic disease therapy. For example, PF4 can be crosslinked to a large carrier protein, e.g., human serum albumin (HSA) or immunoglobulin, by disuccinimidyl suberate (DSS) through free primary amino groups (i.e., lysine E-amino groups or N-terminal α -amino groups; see Montesano et al. [1982] Biochem. Biophys. Res. Comm. 109:7-13).

Purified rPF4 and HSA (10 mg and 100 mg, respectively) were incubated with 25 mM DSS for 4 hours at room temperature. The reaction was terminated by the addition of Tris buffer, pH 8.0 to a final concentration of 100 mM. The resulting composition was a heterogeneous mixture of crosslinked molecules which lacked heparin binding activity, but retained the ability to inhibit HUVEC proliferation. A control sample in which HSA was crosslinked to cytochrome-C did not inhibit HUVEC growth.

Example 15 - Delivery of Toxin Molecules to PF4 Specific Targets

It is sometimes advantageous to use PF4 as the targeting molecule for directing the activity of a toxic agent to a particular cell type. For example, PF4 can be chemically or genetically crosslinked to the toxin ricin A or the diphtheria toxin.

A fusion protein comprised of PF4 and ricin A can be produced recombinantly in a prokaryotic or eukaryotic host. The resulting purified toxin will have the high specificity for endothelial cells or cells in close proximity to endothelial cells, e.g., tumor cells. Alternatively, PF4 and ricin can be linked with cross linkers. DSS can cross link PF4 and ricin A while retaining both PF4 and ricin A activities.

PF4 can also be covalently linked with a cross linker to photoactivatable molecules, for example, hematoporphyrin derivative (HPD). Water soluble carbodiimides (e.g., EDC) are most useful in linking the acid side chains of HPD to the amino groups of PF4. The resulting conjugate will concentrate at sites rich in endothelial cells (such as solid tumors) and can be activated by relatively non-toxic laser or phototherapy focused directly on the tumor site. Activated HPD is known to generate active oxygen species which non-specifically kill nearby cells.

Example 16 - Modification of the Cysteine Residues of rPF4

During preparation, the disulfides of rPF4 are reduced by dithiothreitol (DTT) to free sulfhydryls, but the heparin binding activity is retained. To assess the biological activity of PF4 requires removal of the DTT which, by allowing the disulfide bridges to reform, obscures whether or not they are essential for these activities.

The sulfhydryls of rPF4 were specifically and irreversibly modified by pre-reduction with DTT followed by treatment with fluorescein maleimide (FM). The reduced and purified rPF4 (5 mg in sodium carbonate buffer, pH 8.5 (SCB)) was treated with 10 mg of FM for 3 hours at room temperature. Residual FM was removed by gel filtration in SCB and then dialyzed against the same buffer. The FM-rPF4 partially retained heparin binding activity. When tested in the CAM and endothelial cell proliferation assays, FM-rPF4 exhibited inhibitory activity indicating that neither free sulfhydryls nor correct disulfide bonds are required for the angiostatic activity of PF4.

This FM-modified rPF4 may possess some utility as an alternative endothelial cell labeling or inhibiting compound but, most importantly, it indicates that the cysteine residues of PF4 are also appropriate targets for conjugating or cross linking PF4 to other molecules for diagnostic or therapeutic applications.

Claims

1. Use of a polypeptide conjugate comprising platelet factor 4 (PF4), or an angiogenesis-inhibiting fragment or mutant thereof, conjugated to a second molecule selected from fluorophores, chelators, carrier molecules, antibodies, toxins and cell receptor molecules or complementary ligands thereof, for the manufacture of a medicament for use in inhibiting angiogenesis or endothelial cellular proliferation.
2. Use according to claim 1, wherein the conjugate comprises at least the sequence
Pro-Leu-Tyr-Gln-Glu-Ile-Ile-Gln-Glu-Leu-Leu-Glu-Ser
3. Use according to claim 1, wherein the conjugate comprises rPF4.
4. Use according to any preceding claim, wherein the second molecule is a fluorescent molecule.
5. Use according to claim 4, wherein the second molecule is fluorescein-isothiocyanate.
6. Use according to any of claims 1 to 3, wherein the second molecule is a toxin.
7. Use according to claim 6, wherein the toxin is diphtheria toxin or ricin A.
8. Use according to any of claims 1 to 3, wherein the second molecule is an antibody.
9. Use according to any of claims 1 to 3, wherein the second molecule is a carrier protein.
10. Use according to claim 9, wherein the carrier protein is a human serum albumin.
11. Use according to any of claims 1 to 3, wherein the second molecule is a chelator.
12. Use according to any of claims 1 to 3, wherein the second molecule is non-proteinaceous.
13. Use according to any of claims 1 to 3, wherein the second molecule is photo-activatable.
14. Use according to claim 13, wherein the photo-activatable molecule is a hematoporphyrin derivative.
15. Use according to any preceding claim, wherein the conjugation is by means of a cross-linker.
16. Use according to claim 15, wherein the cross-linker is disuccinimidyl suberate or a water-soluble carbodiimide.
17. Use according to any preceding claim, wherein the conjugation is via the lysine or cysteine residues of the PF4, fragment or mutant.

Patentansprüche

1. Verwendung eines Polypeptid-Konjugats, umfassend Blutplättchen-Faktor 4 (PF4) oder ein Angiogenese-inhibierendes Fragment oder eine Mutante davon, konjugiert an ein zweites Molekül, welches aus Fluorophoren, Chelatoren, Trägermolekülen, Antikörpern, Toxinen und Zellrezeptor-Molekülen oder komplementären Liganden davon ausgewählt ist, zur Herstellung eines Medikaments zur Verwendung bei der Inhibierung von Angiogenese oder Endothelzell-Proliferation.
2. Verwendung nach Anspruch 1, worin das Konjugat mindestens die Sequenz
Pro-Leu-Tyr-Gln-Glu-Ile-Ile-Gln-Glu-Leu-Leu-Glu-Ser
 umfaßt.
3. Verwendung nach Anspruch 1, worin das Konjugat rPF4 umfaßt.

4. Verwendung nach irgendeinem der vorhergehenden Ansprüche, worin das zweite Molekül ein fluoreszierendes Molekül ist.
5. Verwendung nach Anspruch 4, worin das zweite Molekül Fluorescein-Isothiocyanat ist.
6. Verwendung nach irgendeinem der Ansprüche 1 bis 3, worin das zweite Molekül ein Toxin ist.
7. Verwendung nach Anspruch 6, worin das Toxin Diphtherie-Toxin oder Ricin A ist.
8. Verwendung nach irgendeinem der Ansprüche 1 bis 3, worin das zweite Molekül ein Antikörper ist.
9. Verwendung nach irgendeinem der Ansprüche 1 bis 3, worin das zweite Molekül ein Trägerprotein ist.
10. Verwendung nach Anspruch 9, worin das Trägerprotein ein Humanserumalbumin ist.
11. Verwendung nach irgendeinem der Ansprüche 1 bis 3, worin das zweite Molekül ein Chelator ist.
12. Verwendung nach irgendeinem der Ansprüche 1 bis 3, worin das zweite Molekül kein Protein ist.
13. Verwendung nach irgendeinem der Ansprüche 1 bis 3, worin das zweite Molekül photoaktivierbar ist.
14. Verwendung nach Anspruch 13, worin das photoaktivierbare Molekül ein Hämatoporphyrin-Derivat ist.
15. Verwendung nach irgendeinem der vorhergehenden Ansprüche, worin die Konjugation mit Hilfe eines Vernetzungsmittels erfolgt.
16. Verwendung nach Anspruch 15, worin das Vernetzungsmittel Disuccinimidylsuberat oder ein wasserlösliches Carbodiimid ist.
17. Verwendung nach irgendeinem der vorhergehenden Ansprüche, worin die Konjugation über die Lysin- oder Cystein-Reste des PF4, des Fragments oder der Mutante erfolgt.

Revendications

1. Utilisation d'un conjugué polypeptidique comprenant le facteur plaquettaire 4 (PF4), ou un fragment ou mutant de celui-ci qui inhibe l'angiogenèse, conjugué à une seconde molécule choisie parmi les fluorophores, les chélatants, les molécules porteuses, les anticorps, les toxines et les molécules de récepteurs cellulaires ou leurs ligands complémentaires, pour la préparation d'un médicament destiné à être utilisé dans l'inhibition de l'angiogenèse ou de la prolifération des cellules endothéliales.
2. Utilisation selon la revendication 1, dans laquelle le conjugué comprend au moins la séquence
Pro-Leu-Tyr-Gln-Glu-Ile-Ile-Gln-Glu-Leu-Leu-Glu-Ser
3. Utilisation selon la revendication 1, dans laquelle le conjugué comprend rPF4.
4. Utilisation selon l'une quelconque des revendications précédentes, dans laquelle la seconde molécule est une molécule fluorescente.
5. Utilisation selon la revendication 4, dans laquelle la seconde molécule est l'isothiocyanate de fluorescéine.
6. Utilisation selon l'une quelconque des revendications 1 à 3, dans laquelle la seconde molécule est une toxine.
7. Utilisation selon la revendication 6, dans laquelle la toxine est la toxine diphtérique ou la ricine A.
8. Utilisation selon l'une quelconque des revendications 1 à 3, dans laquelle la seconde molécule est un anticorps.

9. Utilisation selon l'une quelconque des revendications 1 à 3, dans laquelle la seconde molécule est une protéine porteuse.
- 5 10. Utilisation selon la revendication 9, dans laquelle la protéine porteuse est une sérumalbumine humaine.
11. Utilisation selon l'une quelconque des revendications 1 à 3, dans laquelle la seconde molécule est un chélatant.
12. Utilisation selon l'une quelconque des revendications 1 à 3, dans laquelle la seconde molécule est non protéique.
- 10 13. Utilisation selon l'une quelconque des revendications 1 à 3, dans laquelle la seconde molécule est photoactivable.
14. Utilisation selon la revendication 13, dans laquelle la molécule photoactivable est un dérivé de l'hématoporphyrine.
- 15 15. Utilisation selon l'une quelconque des revendications précédentes, dans laquelle la conjugaison est accomplie au moyen d'un agent de réticulation.
16. Utilisation selon la revendication 15, dans laquelle l'agent de réticulation est le subérate de disuccinimidyle ou un carbodiimide hydrosoluble.
- 20 17. Utilisation selon l'une quelconque des revendications précédentes, dans laquelle la conjugaison est accomplie par l'intermédiaire des résidus lysine ou cystéine de PF4, d'un fragment ou d'un mutant.

25

30

35

40

45

50

55

Inhibition of Angiogenesis by rPF-4 and Related Peptides

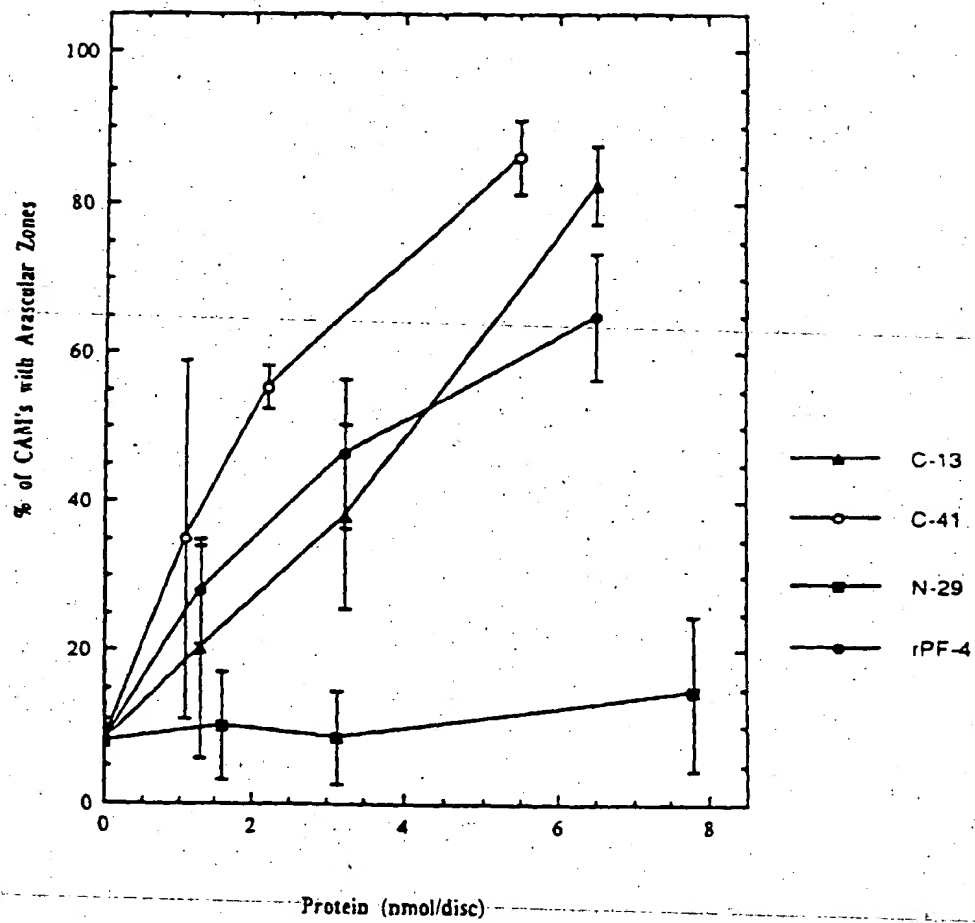


Figure 1

Figure 2.

rPF4 and rPF4-241

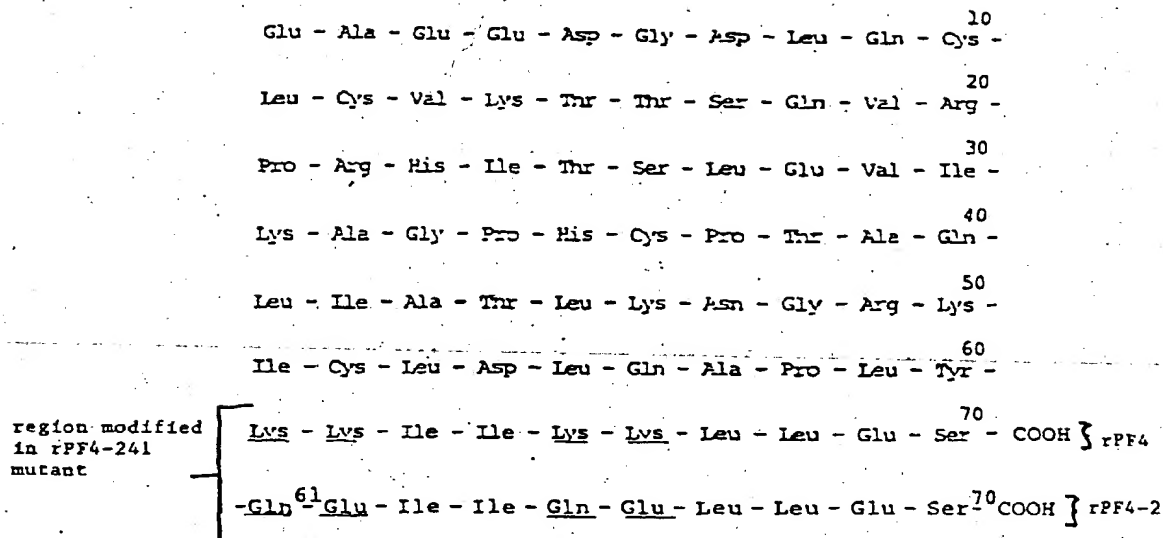
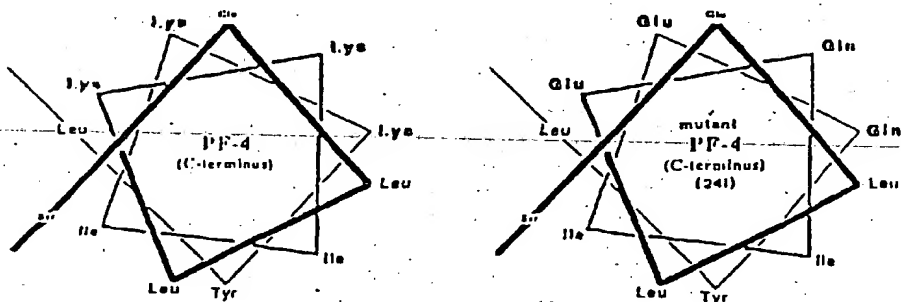


Figure 3

 Axial alpha-helical projections of the
 carboxy-terminus of rPF4 and rPF4-241


Inhibition of Angiogenesis by Native
Sequence and Mutant rPF-4

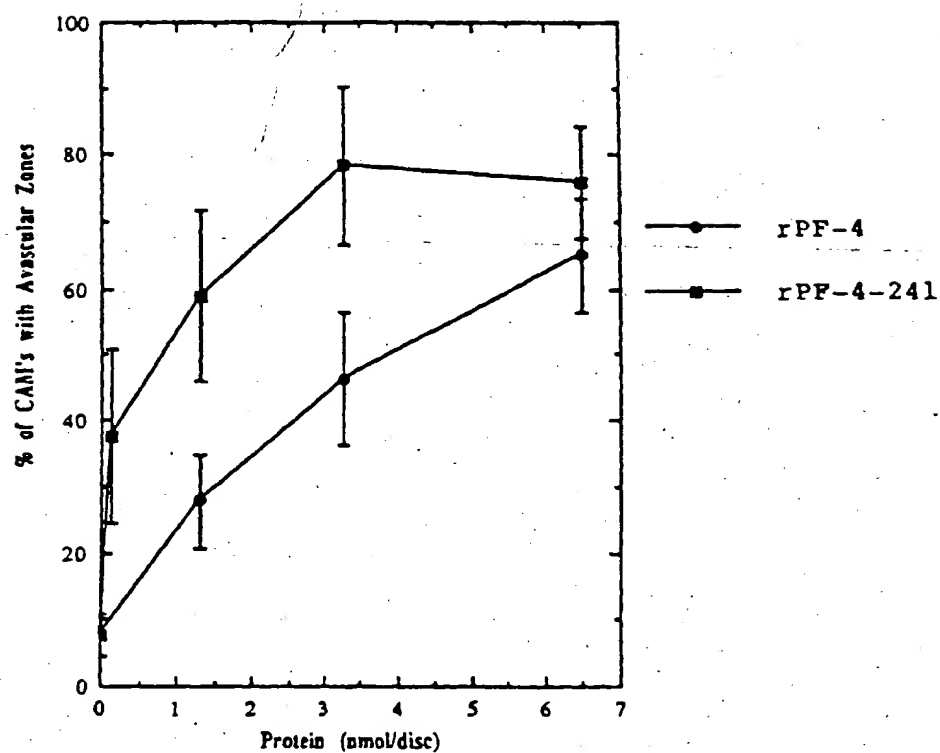
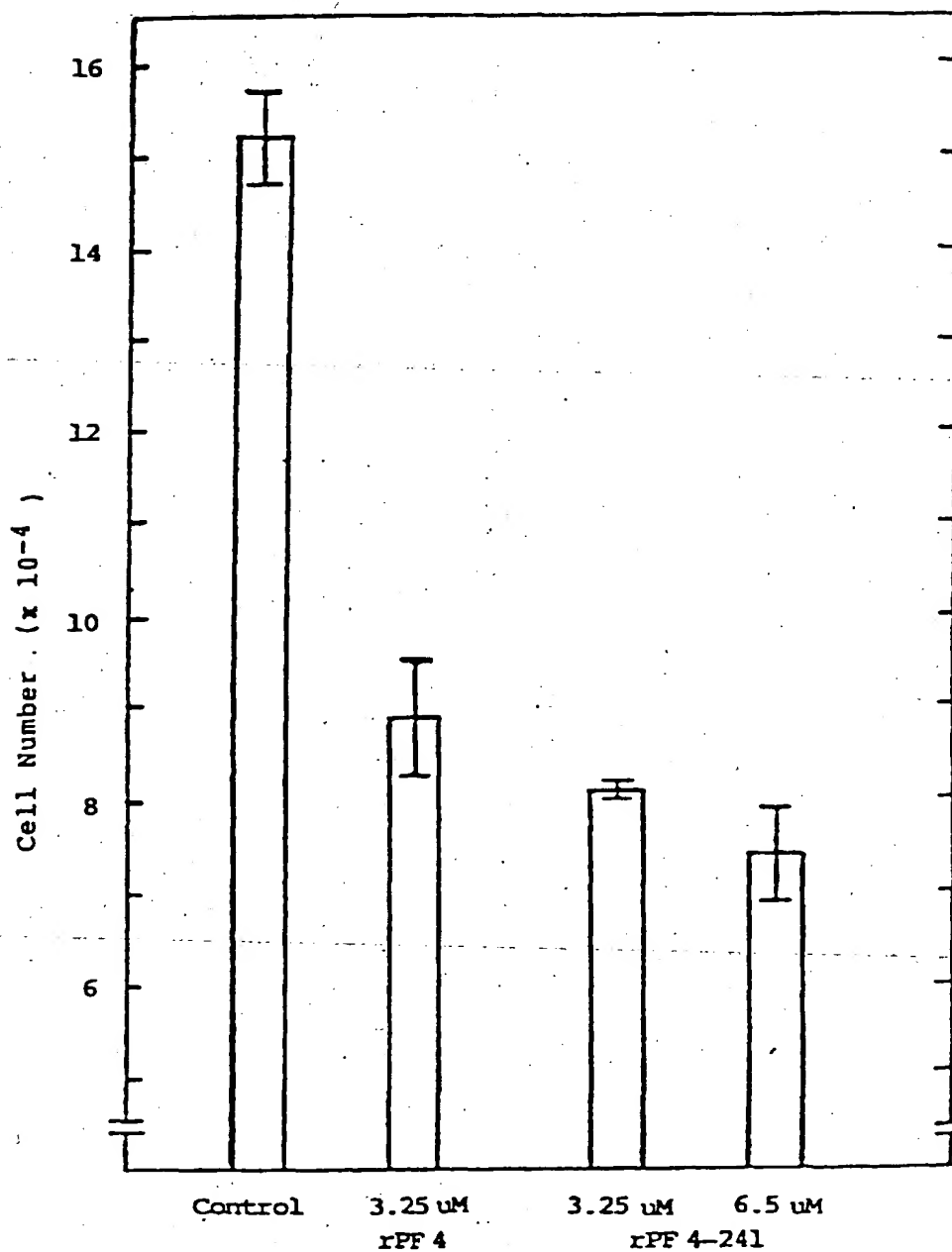


Figure 4

Figure 5

Inhibition of human
endothelial cell proliferation by
rPF 4 and rPF 4-241



Inhibition of Human Umbilical Vein Endothelial Cell Proliferation
by Native rPF4 and Mutant rPF4-241

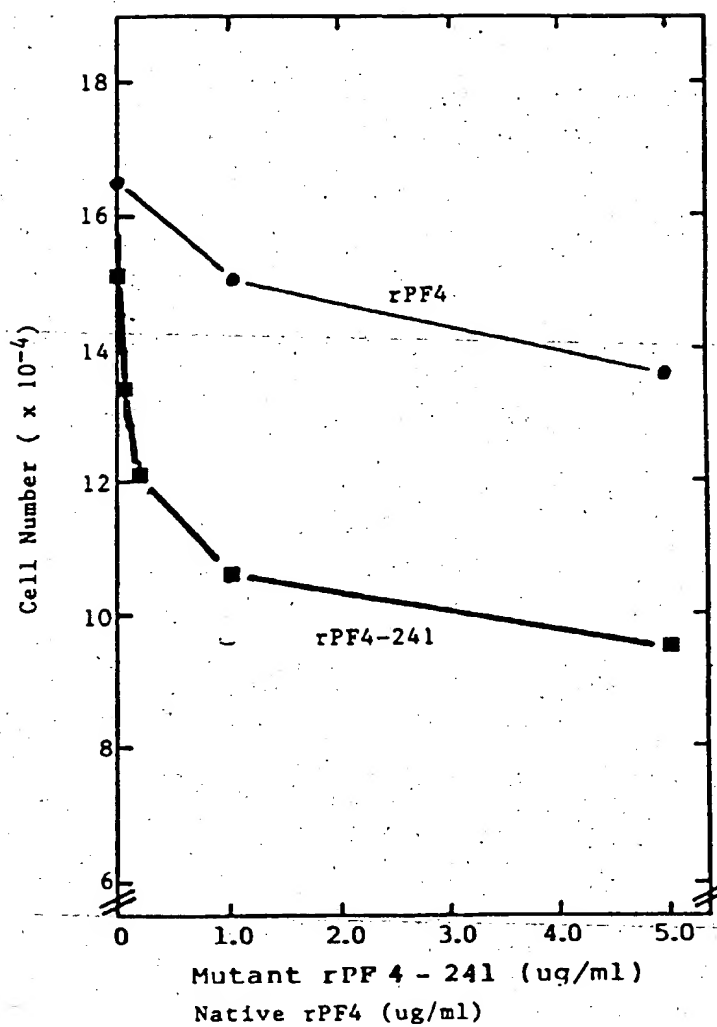


Figure 6

B16 MURINE MELANOMA GROWTH KINETICS

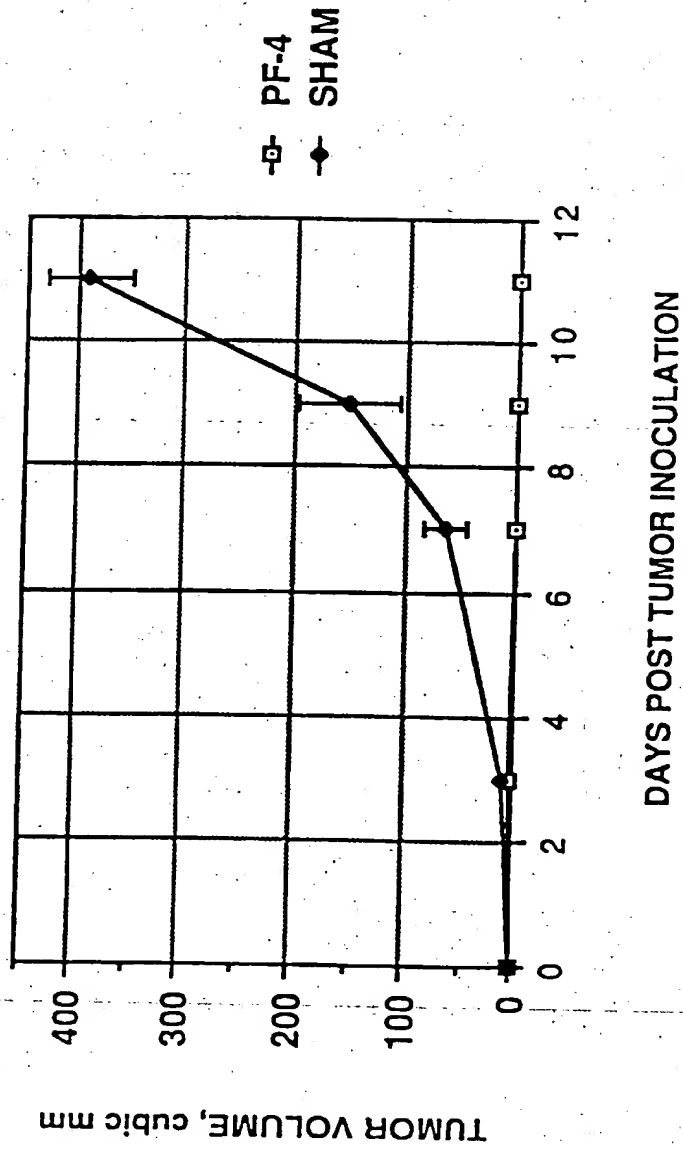


Figure 7

FIGURE 8

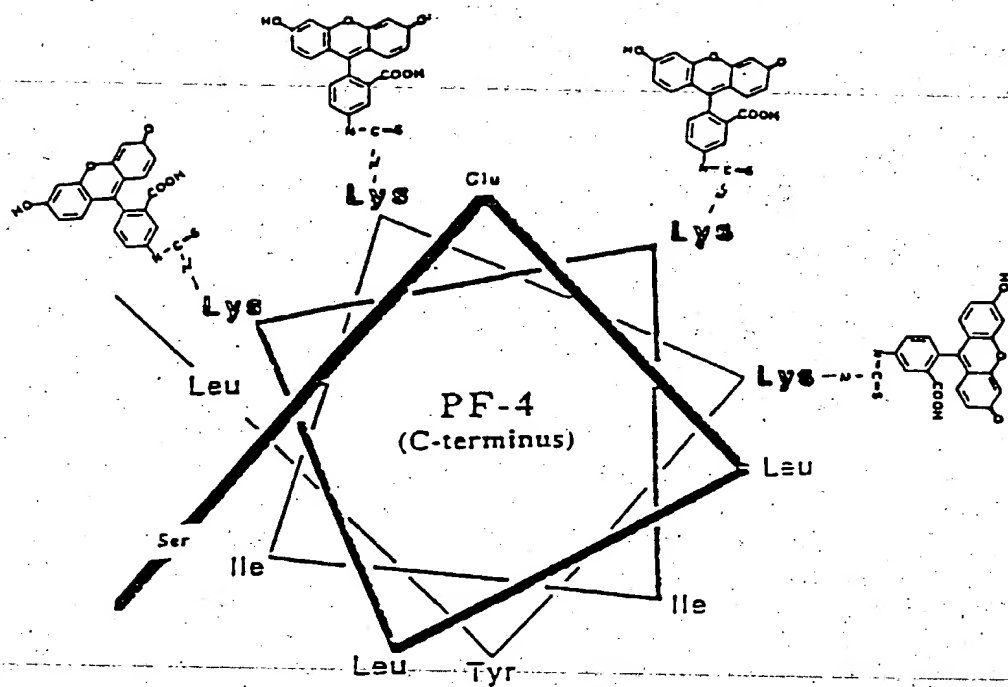
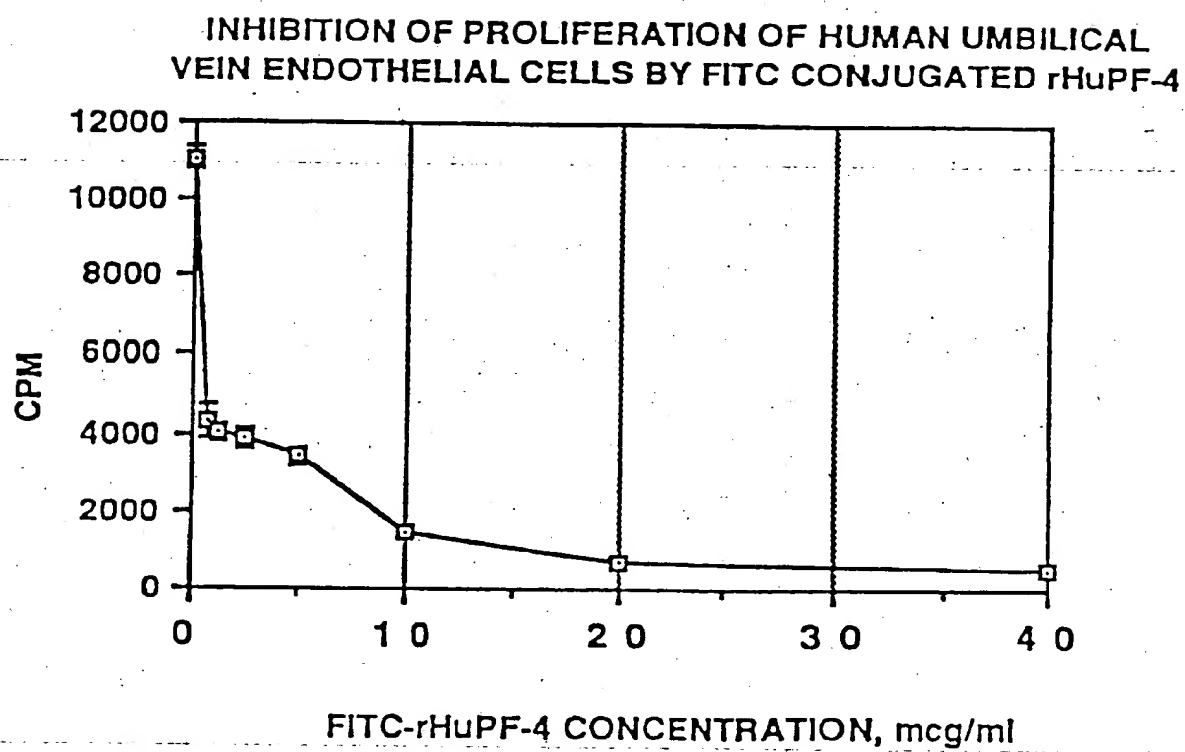


FIGURE 9



**Inhibition of HUVEC Proliferation by
FITC-labeled rPF-4-241**

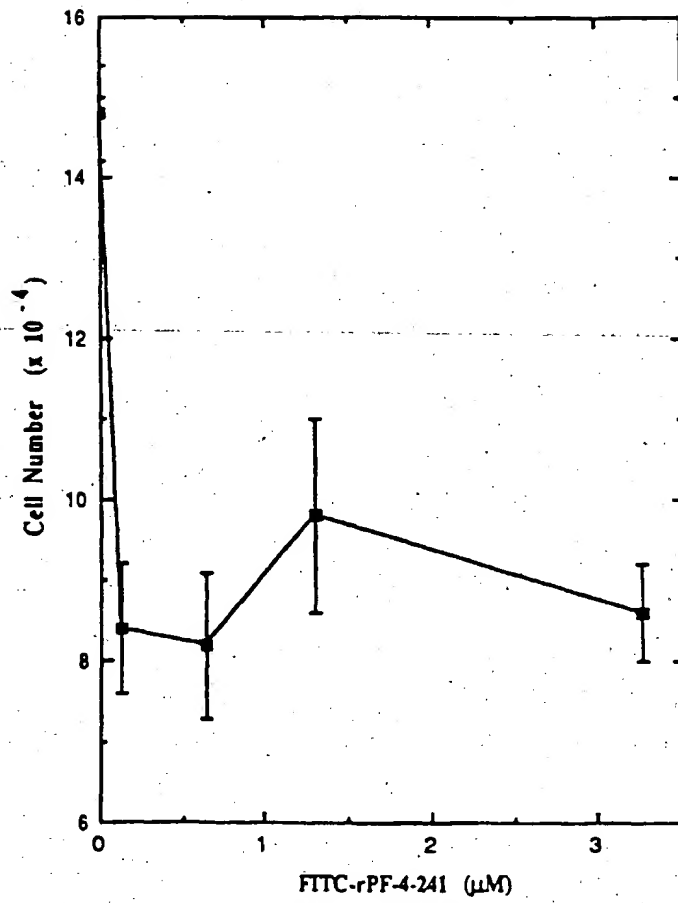


Figure 10

Figure 11

